

REMARKS

Claims 32-42 are now in the case. Claim 42 has been added. Support for the newly added claim can be found in the claims as originally filed. No new matter has been added.

OBJECTION OF THE SPECIFICATION

The specification is objected to because the specification contains browser-executable code. The Office requests that all use of the prefix “www.” be removed.

Applicant has provided the requested amendments as is indicated above.

REJECTION UNDER 35 U.S.C. §101 and §112, First Paragraph

Claims 1-11, 14-23 and 26 remain rejected under 35 U.S.C. §101 and §112, first paragraph, on the basis that these claims allegedly lack patentable utility, and that one skilled in the art would therefore not know how to use the invention.

Applicant respectfully traverses this rejection. However, merely to advance prosecution of the claims towards allowance claims 1-11, 14-23 and 26 have been cancelled. Applicant reserves the right to pursue the subject matter of the cancelled claims in continuation applications. New claims 32-42 are directed towards an isolated polypeptide comprising SEQ ID NO: 12 and polypeptide comprising amino acid residues 389 through 491 of SEQ ID NO:12.

Applicant's invention is a new member of the ADAM family of proteins. One of the main distinguishing characteristics of this family is the presence of an integrin-binding disintegrin domain. Disintegrin domains have been identified in cellular proteins from both invertebrates and vertebrates. All ADAMs contain a disintegrin domain, approximately 80 amino acid residues, with 15 highly conserved cysteine residues (page 2, line 8-9). ADAMs are recognized as potential ligands for integrins and have been shown to bind integrins.

Integrins are a family of cell adhesion receptors that bind various ligands including extracellular matrix proteins and other cell surface molecules. Integrins are involved in cell-to-cell adhesion, cell-to-matrix adhesion, and inflammatory responses (page 1, lines 21-31). Integrins are found on the surface of various cells, such platelets, fibroblasts, endothelial, muscle, neuronal, bone, sperm, egg, and tumors cells. Integrins play an important role in biological processes including embryonic development, platelet aggregation, immune

reactions, tissue repair and remodeling, bone resorption, and tumor invasion and metastasis and are important targets for therapeutic intervention in human disease.

The involvement of integrins in cell-to-cell adhesion, cell-to-matrix adhesion, and inflammatory responses makes the binding activity of disintegrin domains a very useful as a tool. Binding of disintegrin domains to integrins has been shown to be responsible for such cell-cell interactions as egg-sperm fusion and muscle cell fusion, for example (page 2 lines 20-25). Agents comprising a disintegrin domain can be used to disrupt normal adhesion processes by inhibiting the binding of cell surface integrins to their ligands. For example, soluble disintegrin domains can be used to inhibit the interaction of integrins and their ligands and exploit this anti-adhesive activity. Target specific inhibitory or agonistic agents are well known and routinely relied upon and are eagerly sought by those in the art.

Of the known ADAMS, the largest group is predominately expressed in testis and is thought to be involved in spermatogenesis and fertilization (page 2 lines 30-39). Fertilin- α /ADAM1 and fertilin- β /ADAM2 are required for sperm-egg function (page 2, lines 20 and 43). Mammalian fertilization involves a cascade of cell-cell and cell-matrix interactions. Disintegrin domains of fertilins are found on the sperm surface are involved in binding and fusion with integrins on the egg plasma membrane.

“Fertilin is the prototype of a growing and widely distributed family (ADAMs) of membrane proteins that possess an integrin ligand domain with the disintegrin motif... Hence, a direct interaction between ADAMs and integrins might represent a novel type of cell-cell interaction used not only for sperm-egg binding, but other important cell-cell recognition ... and signaling events.” (see page 1101, last full paragraph, left column, Almeida et al., Cell 81: 1095-1104, 1996, emphasis added, a copy is provided).

Utility under 35 U.S.C. §101 is a minimal threshold issue that can be satisfied by a showing of any use that is “credible,” “specific” and “substantial” (MPEP §2107). A small degree of utility is sufficient. Thus, as a matter of Patent Office practice, a specification that provides disclosure of a utility that corresponds in scope to the subject matter sought to be patented and that is substantial, credible, and specific must be taken as sufficient to satisfy the utility requirement of 35 U.S.C. §101 for the entire claimed subject matter unless there is reason for one skilled in the art to question the objective truth of the statement of utility.

SVPH-1a is an ADAM protein and like all other ADAMs it has a disintegrin domain that binds integrins. Disintegrin domains are known to bind to integrins, the usefulness of such integrin-binding proteins is known. Like the majority of known ADAMs, SVPH-1a is associated with and is predominantly expressed on testis tissue. The integrin binding by disintegrin domains of testis-expressed ADAM proteins has been shown to be a component in the process of fertilization. The integrin binding capability of ADAM proteins is recognized as useful and desirable.

SVPH-1a and its disintegrin domain are useful, in part, due to the specificity of the disintegrin domain as an integrin binding partner. The value of such integrin binding proteins is recognized as credible and one of skill in the art appreciates the usefulness of disintegrin domain-containing ADAM proteins, including SVPH-1a. This use is also substantial in that there are real world uses for proteins that bind integrins. As discussed above, integrins are involved in cell-cell and cell-matrix adhesion and as such play an important role in biological processes and are important targets for therapeutic intervention in human disease. Mammalian fertilization involves a cascade of cell-cell and cell-matrix interactions. The disintegrin domains of similarly expressed ADAMs are involved in binding and fusion with integrins on the egg plasma membrane. SVPH-1a is predominantly expressed in testis tissue. Like those skilled in the art, Applicant appreciated the usefulness of an integrin-binding protein, particularly one that is predominantly expressed in a critical tissue and anticipated use of the disintegrin domain and its integrin binding properties. Soluble disintegrin domain proteins have further use for binding integrins expressed on other tissues.

The use of such integrin binding proteins would be readily apparent to one of skill in the art and immediate application of such proteins, for uses such as those described above, would be recognized as credible, specific and substantial.

Therefore, for at least the reasons presented above, Applicant has asserted in the specification a specific, substantial, and credible use for compositions of matter of the invention, and withdrawal of the rejection of claims 1-11, 14-23 and 26 under 35 U.S.C. §101 and §112, first paragraph, is respectfully requested.

REJECTION UNDER 35 U.S.C. §112, First Paragraph (written description)

Claims 1, 3-10, 14-22 and 26 remain rejected under 35 U.S.C. §112, First Paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the invention was filed, had possession of the claimed invention.

Applicant respectfully traverses these grounds for rejection. As discussed above, claims 1, 3-10, 14-22 and 26 have been cancelled. Applicant reserves the right to pursue the cancelled matter in future continuation applications. Therefore for these reasons Applicant believes that the rejection is moot and withdrawal of the rejection is respectfully requested.

REJECTION UNDER 35 U.S.C. §112, First Paragraph (enablement)

Claims 1, 3-10, 14-22 and 26 remain rejected under 35 U.S.C. §112, First Paragraph, because the specification, while being enabling for the preparation of a polypeptide having the amino acid sequence of the elected SEQ ID NO:12 having a disintegrin activity, does not reasonably enable preparation of amino acid sequences having disintegrin activity that diverge from the amino acid sequence of SEQ ID NO:12 by unlimited amino acid substitutions, deletions and insertions, or combinations thereof anywhere within SEQ ID NO:12.

Applicant respectfully traverses these grounds for rejection. As discussed above, claims 1, 3-10, 14-22 and 26 have been cancelled. Therefore for these reasons Applicant believes that the rejection is moot and withdrawal of the rejection of is respectfully requested.

REJECTION UNDER 35 U.S.C. §112, Second Paragraph

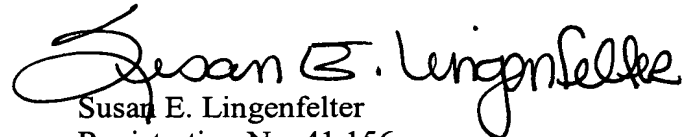
Claims 1-11, 14-23, and 26 remain rejected under 35 U.S.C. §112, Second Paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

Applicant respectfully traverses these grounds for rejection. As discussed above, claims 1-11, 14-23, and 26 have been cancelled. Therefore for these reasons Applicant believes that the rejection is moot and withdrawal of the rejection of is respectfully requested.

CONCLUSION

Applicant submits that the presented claims are in condition for allowance. A favorable action is earnestly requested. Applicant's attorney invites the Examiner to call her at the number below if any issue remains outstanding.

Respectfully submitted,



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Mouse Egg Integrin $\alpha 6 \beta 1$ Functions as a Sperm Receptor

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Summary

Binding between sperm and egg plasma membranes is an essential step in fertilization. Whereas fertilin, a mammalian sperm surface protein, is involved in this crucial interaction, sperm receptors on the egg plasma membrane have not been identified. Because fertilin contains a predicted integrin ligand domain, we investigated the expression and function of integrin subunits in unfertilized mouse eggs. Polymerase chain reactions detected mRNAs for $\alpha 5$, $\alpha 6$, αv , $\beta 1$, $\beta 3$, and $\beta 5$. Immunofluorescence revealed $\alpha 6 \beta 1$ and $\alpha v \beta 3$ on the plasma membrane. GoH3, a function-blocking anti- $\alpha 6$ monoclonal antibody, abolished sperm binding, but a nonfunction-blocking anti- $\alpha 6$ monoclonal antibody, a function-blocking anti- $\alpha v \beta 3$ polyclonal antibody, and an RGD peptide had no effect. Somatic cells bound sperm avidly, but only if they expressed $\alpha 6 \beta 1$. A peptide analog of the fertilin integrin ligand domain inhibited sperm binding to eggs and $\alpha 6 \beta 1^+$ cells and diminished GoH3 staining of eggs. Our results indicate a novel role for the integrin $\alpha 6 \beta 1$ as a cell–cell adhesion receptor that mediates sperm–egg binding.

Introduction

The process of mammalian fertilization involves a cascade of cell–cell and cell–matrix interactions. First, sperm bind to the zona pellucida, a large extracellular matrix surrounding the egg. This interaction triggers the acrosome reaction that, in turn, allows sperm to penetrate the zona. Finally, sperm approach, bind to, and fuse with the egg plasma membrane. While details of the molecular interactions between sperm and the egg zona pellucida are beginning to emerge, little is known about the molecular basis

for the essential plasma membrane binding and fusion reactions that initiate embryonic development (Myles, 1993; Wassarman, 1990; Yanagimachi, 1994).

We have recently described and characterized fertilin α/β (previously called PH-30 α/β), a complex of sperm surface antigens that is involved in binding and fusion between sperm and egg plasma membranes (Blobel et al., 1990, 1992; Myles et al., 1994; Primakoff et al., 1987; Wolfsberg et al., 1993). Fertilin shares similarities with certain viral adhesion/fusion glycoproteins including membrane topology, proteolytic processing from larger precursors, and predicted binding and fusion domains (White, 1992).

The predicted binding domain of fertilin β shares high sequence similarity with a family of soluble disintegrins found in snake venoms (Blobel and White, 1992; Wolfsberg et al., 1993, 1995). Members of the two snake venom disintegrin subfamilies, PII and PIII (Hite et al., 1994), function as integrin ligands as evidenced by their ability to disrupt cell–matrix interactions (Blobel and White, 1992; Gould et al., 1990; Kini and Evans, 1992; Usami et al., 1994). PII disintegrins display a tripeptide (e.g., RGD or KGD) at the tip of a 13 amino acid loop that interacts with integrins, notably $\alpha \text{IIb} \beta 3$ on platelets (Adler et al., 1991; Saudek et al., 1991). PIII disintegrins are larger, possessing a cysteine-rich domain COOH-terminal to their disintegrin domains (Fox and Bjarnason, 1995; Hite et al., 1994). In place of the 13 amino acid loop, PIII disintegrins possess a 14 amino motif with sequences such as ESEC and RSEC in lieu of the canonical RGD of the PII disintegrins. In contrast with the soluble snake venom disintegrins, fertilin β is membrane anchored, suggesting that it is the prototype of a novel family of cell adhesion molecules. Support for this hypothesis has come from our recent description of a large family of fertilin homologs, which we have termed ADAMs (Wolfsberg et al., 1993, 1995). The disintegrin domains of all known ADAMs resemble those of PIII snake disintegrins (Fox and Bjarnason, 1995; Wolfsberg et al., 1995).

The observation that fertilin β possesses a disintegrin domain (Blobel et al., 1992) led us to predict that an integrin on the egg plasma membrane could serve as a sperm receptor. Several lines of indirect evidence have supported this notion. First, peptide analogs of the disintegrin domain from guinea pig fertilin β inhibit fertilization in vitro (Myles et al., 1994). Second, integrins have been detected on the surface of mammalian eggs (Fusi et al., 1993; Hierck et al., 1993; Sutherland et al., 1993; Tarone et al., 1993). Third, divalent cations are required for sperm–egg fusion (Yanagimachi, 1994). Fourth, RGD-containing peptides have been reported to inhibit fusion of human and hamster sperm to golden hamster eggs (Bronson and Fusi, 1990). Despite these indications, there is currently no evidence implicating a specific integrin as a sperm receptor.

In the present study, we provide a direct test of the hypothesis that a specific integrin serves as a sperm receptor. After characterizing major integrins present on the

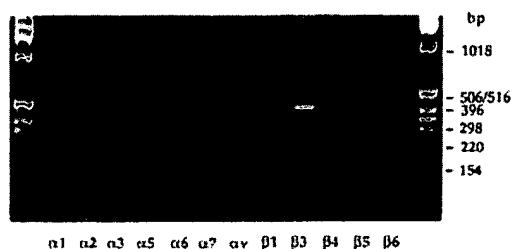


Figure 1. PCR Analysis of Egg mRNA Using Primers for Integrin Subunits

mRNA from mature unfertilized mouse eggs was reverse transcribed and subjected to PCR with primer pairs designed to amplify the indicated integrin subunits. The results shown were obtained from one preparation of egg cDNA. For each set of primers, cDNA from E13.5 mouse embryos was used as a positive control (data not shown). Positions of DNA size markers are labeled to the right of the figure.

plasma membrane of unfertilized mouse eggs, a combination of antibody inhibition, peptide inhibition, and somatic cell transfection experiments has led us to conclude that the integrin $\alpha 6 \beta 1$ serves as a murine sperm receptor and that the disintegrin domain of fertilin is involved in $\alpha 6 \beta 1$ -mediated sperm binding.

Results

We predicted that an integrin on the surface of the egg plasma membrane serves as a sperm receptor based on the observation that fertilin, a sperm surface protein involved in binding and fusion, contains a potential integrin ligand domain (Biobel et al., 1992). Here, we have explored this hypothesis by asking four questions. Which integrins are present on the surface of unfertilized mouse eggs? Are any of these integrins involved in binding sperm? Do candidate integrins also bind sperm when expressed in somatic cells? Is fertilin involved in integrin-mediated sperm binding?

Expression of Integrin Subunits in the Mouse Egg

At present count, 14 α and 8 β integrin subunits have been described, and 14 have been sequenced from the mouse. To begin to identify which integrin subunits are expressed in mouse eggs, we conducted polymerase chain reaction (PCR) assays to determine which integrin subunit messages are present, and then indirect immunofluorescence to determine which protein subunits are expressed on the egg surface. For the PCR analysis, we used primer pairs that would amplify 13 mouse integrin subunits. Our primer set comprised all sequenced mouse integrin subunits except $\beta 2$, which is thought to be expressed exclusively in the immune system. The results, shown in Figure 1, revealed that mRNAs for three α and three β subunits are expressed in the mouse egg: $\alpha 5$, $\alpha 6$ (A and B), αv , $\beta 1$, $\beta 3$, and $\beta 5$. Conversely, mRNAs for $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$ (data not shown), $\alpha 7$, $\beta 4$, and $\beta 6$ subunits are not expressed.

We next employed polyclonal and monoclonal antibodies (MAbs) to determine which of the detected egg integrin mRNAs ($\alpha 5$, $\alpha 6$, αv , $\beta 1$, $\beta 3$, $\beta 5$) are expressed as proteins

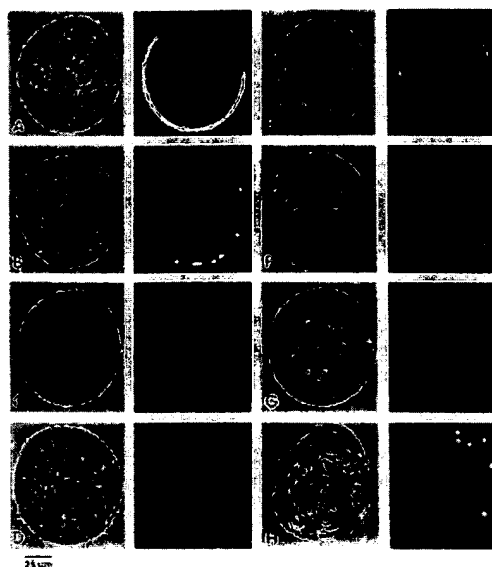


Figure 2. Indirect Immunofluorescence of Integrins on the Surface of Unfertilized Mouse Eggs

Live (A–B and D–G) or fixed/permeabilized (C) eggs were subjected to indirect immunofluorescence. Primary antibodies were as follows: pan-integrin polyclonal antiserum, which recognizes $\alpha 3 \beta 1$, $\alpha 5 \beta 1$, $\alpha 6 \beta 1$, and $\alpha v \beta 3$ (A); polyclonal antiserum against $\alpha v \beta 3$ (B); polyclonal antiserum against a peptide from the cytoplasmic domain of $\alpha 3$ (C); normal rabbit serum (D); MAb GoH3, which recognizes $\alpha 6$ (E); MAb J1B5, which recognizes $\alpha 6$ (F); MAb anti- $\alpha 5$, which recognizes $\alpha 5$ (G). In (H), live (neither fixed nor permeabilized) early mouse blastocysts were labeled with the MAb anti- $\alpha 5$, as in (G). Scale bar = 25 μ m.

on the surface of the mouse egg. A polyclonal "pan-integrin" antiserum that recognizes and blocks the function of $\alpha 3 \beta 1$, $\alpha 5 \beta 1$, $\alpha 6 \beta 1$, and $\alpha v \beta 3$ heterodimers (Buck and Horowitz, 1987) showed intense labeling of the microvillar surface and weak labeling of the smooth-surfaced cortical granule-free region that overlies the metaphase II spindle (Figure 2A). A polyclonal antiserum (Figure 2B) that recognizes and blocks function of the $\alpha v \beta 3$ integrin heterodimer (Ruoslahti and Pierschbacher, 1987) gave a similar labeling pattern, as did two MAbs against $\alpha 6$: GoH3 (Figure 2E), which blocks the function of $\alpha 6$ (Sonnenberg et al., 1988), and J1B5 (Figure 2F), which does not (Damsky et al., 1992). A function-blocking MAb against the $\alpha 5$ integrin subunit (5H10; Pharmingen, San Diego, California) did not label either unpermeabilized (Figure 2G) or saponin-permeabilized (data not shown) eggs, although it labeled early blastocyst stage embryos (Figure 2H) (Sutherland et al., 1993). A polyclonal antiserum against a cytoplasmic domain peptide of the $\alpha 3$ integrin subunit (Hynes and Marcantonio, 1989) did not stain permeabilized eggs (Figure 2C).

Collectively, these results indicate that, of the six integrin subunits whose mRNA we detected by PCR (Figure 1), the $\alpha 6$, αv , $\beta 1$, and $\beta 3$ proteins are present on the surface of the mouse egg at levels detectable by our methods. The $\alpha 6$ integrin subunit is known to form heterodimers with either $\beta 1$ or $\beta 4$ (Hemler et al., 1989, 1988; Kajiji et

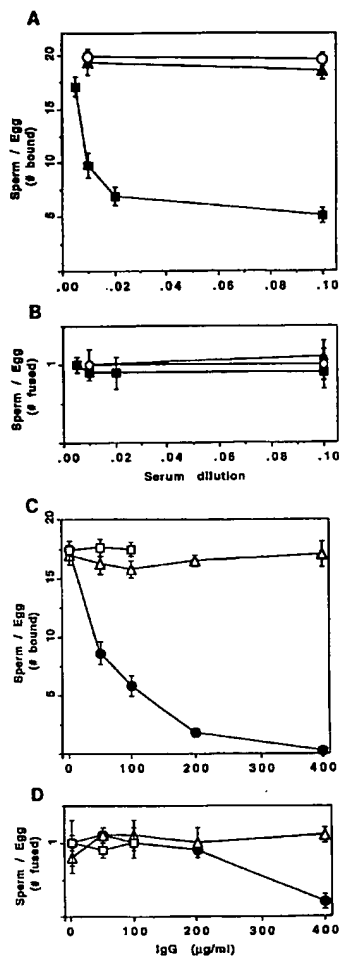


Figure 3. Effects of Polyclonal and Monoclonal Anti-Integrin Antibodies on Binding and Fusion of Mouse Sperm and Eggs

Eggs were pretreated with polyclonal (A and B) or monoclonal (C and D) antibodies, incubated with sperm, washed, and analyzed for sperm binding (A and C) and fusion (B and D). Polyclonal antibodies (A and B): pan-integrin, closed squares; anti- $\alpha\text{v}\beta 3$, closed triangles; normal rabbit serum, open circles. MAbs (C and D): GoH3, closed circles; J1B5, open triangles; anti- $\alpha 5$, open squares. Each experiment included at least 20 eggs per data point and was repeated at least three times. Acrosome-reacted (50%–75% of population) and acrosome-intact sperm bound equally well to zona-free eggs. GoH3 did not preferentially inhibit binding of acrosome-intact or acrosome-reacted sperm (data not shown). A representative experiment is shown. For most experiments, eggs were obtained from ovaries and matured in vitro. Similar results were obtained with in vivo matured, zona-free eggs obtained from ovulated egg masses.

al., 1989; Sonnenberg et al., 1990). Because there is no $\beta 4$ mRNA in unfertilized eggs (Figure 1) or $\beta 4$ protein on the surface of mouse eggs (Hierck et al., 1993), $\alpha 6$ is most likely present as the $\alpha 6\beta 1$ heterodimer. The αv integrin subunit is known to form heterodimers with either $\beta 1$, $\beta 3$, $\beta 5$, $\beta 6$, or $\beta 8$ (Nishimura et al., 1994; Ruoslahti, 1991). Of these, we have confirmed the presence of $\alpha\text{v}\beta 3$ protein

on the surface of mouse eggs. Based on our PCR analysis (Figure 1), the egg may also express $\alpha\text{v}\beta 1$ and $\alpha\text{v}\beta 5$ (but see below). Thus, our results indicate that the integrins $\alpha 6\beta 1$, a laminin receptor (de Curtis et al., 1991; Shaw et al., 1990; Sonnenberg et al., 1988), and $\alpha\text{v}\beta 3$, a fibronectin/vitronectin receptor (Ruoslahti, 1991), are present on the surface of mature unfertilized mouse eggs.

Anti-Integrin Antibodies Inhibit Sperm-Egg Binding

We next assessed whether egg integrins are involved in sperm-egg binding by determining the effects of anti-integrin antibodies on sperm binding to zona-free eggs. The polyclonal pan-integrin antibody, which reacts with $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, and $\alpha\text{v}\beta 3$, inhibited sperm binding to zona-free mouse eggs in a dose-dependent fashion (Figure 3A, closed squares). With a 1:10 dilution of antiserum, binding was inhibited by ~75%, from ~20 to ~5 sperm bound per egg. Despite this potent inhibition of binding, the rate of sperm-egg fusion was not affected: ~1 sperm fused per egg whether there were ~20 or ~5 sperm bound (Figure 3B, closed squares), indicating that a tight block to polyspermy is maintained in this in vitro system.

Because we have not detected the $\alpha 3$ subunit in mouse eggs at either the mRNA (Figure 1) or protein (Figure 2C) level, nor any $\alpha 5$ subunit protein on the surface of mouse eggs (Figure 2G), the inhibitory action of the pan-integrin antibody (Figure 3A) suggested a role for either $\alpha\text{v}\beta 3$ or $\alpha 6\beta 1$ in sperm binding. To assess the involvement of $\alpha\text{v}\beta 3$, we employed an anti- $\alpha\text{v}\beta 3$ polyclonal antiserum that blocks outgrowth of mouse embryos (Sutherland et al., 1993). High concentrations (e.g., 1:10 dilution) of this antibody had no effect on sperm binding (Figure 3A, closed triangles) or fusion (Figure 3B, closed triangles), suggesting that despite its presence on the egg surface (Figure 2B), $\alpha\text{v}\beta 3$ is not involved in sperm binding. To substantiate this claim, we tested the effects of the peptide GRGDS on sperm binding. GRGDS is a well-known inhibitor of $\alpha\text{v}\beta 1$, $\alpha\text{v}\beta 3$, $\alpha\text{v}\beta 5$, and $\alpha 5\beta 1$ integrins (Cheresh et al., 1989; Pytela et al., 1985); it does not inhibit the function of $\alpha 6\beta 1$ (Mercurio and Shaw, 1991). GRGDS did not inhibit either sperm binding to (Table 1) or fusion with (data not shown) zona-free eggs. The lack of effect of GRGDS indicates that neither $\alpha\text{v}\beta 3$ (Figure 2B), $\alpha\text{v}\beta 1$, nor $\alpha\text{v}\beta 5$ (if the latter two are present on the egg surface) are involved in binding between sperm and egg plasma membranes. As

Table 1. Effects of RGD and RGE Peptides on Sperm Binding to Mouse Eggs

Peptide	Concentration (μM)	Sperm Bound per Egg \pm SD
None	—	12.6 \pm 5.9
GRGDS	100	11.1 \pm 5.2
GRGDS	250	12.6 \pm 7.6
RGES	100	12.9 \pm 5.8
RGES	250	12.2 \pm 6.3

Eggs were preincubated with the indicated concentration of peptide for 30 min prior to insemination. Sperm were incubated with eggs for 1 hr prior to washing and counting. 20–30 eggs were used per sample.

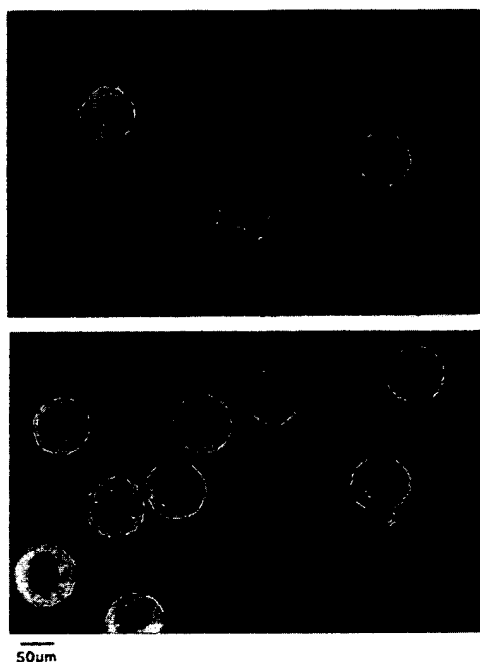


Figure 4. Effect of GoH3 on Sperm Binding to Mouse Eggs. Phase-contrast micrographs of sperm binding to mouse eggs. (A) Untreated. (B) Treated with 200 µg/ml GoH3. Scale bar = 50 µm.

$\alpha v\beta 3$ is present on unfertilized eggs and then continuously in preimplantation embryos, it likely plays a different role in early mouse development (Sutherland et al., 1993).

Collectively, these results point to a key, but not necessarily exclusive, role for the integrin $\alpha 6\beta 1$ in binding mouse sperm to mouse eggs. To explore further the involvement of $\alpha 6\beta 1$, we employed the previously described function-blocking and nonfunction-blocking anti- $\alpha 6$ MABs (Figures 2E and 2F). The function-blocking MAB GoH3 inhibited sperm binding to eggs in a dose-dependent fashion (Figure 3C, closed circles). A visual demonstration of the inhibitory action of GoH3 is shown in Figure 4. Despite its potent inhibition of sperm binding, GoH3 did not inhibit fusion per se. Fusion was only inhibited when GoH3 reduced sperm binding to, on average, less than one sperm per egg (Figure 3D). This suggests, as above (Figure 3B), that fusion is an efficient consequence of having even one sperm bound. In contrast with GoH3, high concentrations of the nonfunction-blocking MAB J1B5 had no effect on sperm binding (Figure 3C, open triangles) or fusion (Figure 3D, open triangles). A function-blocking MAB against the $\alpha 5$ integrin subunit also had no effect on binding or fusion (Figures 3C and 3D, respectively, open squares).

Sperm Bind to Cultured Cells That Express $\alpha 6\beta 1$

The results presented thus far strongly suggest that the mouse egg integrin $\alpha 6\beta 1$ is critically involved in binding mouse sperm (Figure 3C). To corroborate this finding, we determined whether mouse sperm bind to cultured cells

that express $\alpha 6\beta 1$ and compared this binding with that of cells that lack either $\alpha 6$ or $\beta 1$.

F9 (mouse) embryonal carcinoma cells express several integrins, including $\alpha 6\beta 1$, on their surface. The $\beta 1$ integrin subunit in these cells was obliterated by a triple knockout homologous recombination approach, generating a cell line referred to as F9 TKO. As a result, no $\beta 1$ integrins (e.g., $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$) are expressed at the surface of F9 TKO cells, but other integrins such as $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 6\beta 4$ remain (Stephens et al., 1993). Sperm bound avidly to F9 cells, whereas binding to the F9 TKO cells was significantly (>75%) reduced (Figure 5A). Sperm binding to F9 cells occurred via sperm heads and resisted repeated washes. Thus, lack of $\beta 1$ integrins on the surface of F9 TKO cells correlates well with diminished sperm binding. Fluorescence-activated cell sorting analysis indicated that F9 cells express approximately 10-fold more $\alpha 6$ on their surface than the F9 TKO cells (see also Stephens et al., 1993). Hence, $\alpha 6\beta 1$ is likely the integrin primarily responsible for sperm binding to F9 cells. To test this possibility, we assessed the effects of GoH3, the function-blocking anti- $\alpha 6$ MAB, on sperm binding to F9 cells. Consistent with our results on mouse eggs (Figure 3C), GoH3 inhibited sperm binding to F9 cells, while its nonfunction-blocking counterpart had little effect (Figure 5B). Thus, our results with F9 and F9 TKO cells support a role for the integrin $\alpha 6\beta 1$ in sperm binding.

Having demonstrated differential sperm binding to cultured cells that do and do not express $\beta 1$ integrins at their surface, we next compared sperm binding to cultured cells that do and do not express $\alpha 6$ integrins. P388D1 mouse macrophages do not express $\alpha 6\beta 1$ on their surface because they do not express $\alpha 6$ mRNA or protein (Shaw et al., 1993). We therefore predicted that P388D1 cells would not bind sperm, whereas transfectants expressing $\alpha 6$ would. To test this, we compared sperm binding to mock-, $\alpha 6A$ -, and $\alpha 6B$ -transfected P388D1 cells. The A and B subunits of $\alpha 6$ differ only in the sequences of their cytoplasmic tails (Hogervorst et al., 1991; Tamura et al., 1991). Minimal sperm binding occurred to the mock (neo) transfected cells that, by analogy with other macrophages (Hemler, 1990), likely express $\alpha 3\beta 1$, $\alpha 4\beta 1$, and $\alpha 5\beta 1$ at their surface, but do not express $\alpha 6\beta 1$ (Shaw et al., 1993). Conversely, significantly enhanced (approximately 10-fold) sperm binding occurred to both the $\alpha 6A$ and $\alpha 6B$ transfectants (Figures 5C–5F). As seen with the F9 cells, sperm binding to $\alpha 6$ -transfected P388D1 cells was stable to repeated washings and occurred via sperm heads. Consistent with our prediction, the function-blocking anti- $\alpha 6$ MAB GoH3 inhibited sperm binding to $\alpha 6B$ -transfected P388D1 cells in a dose-dependent fashion, whereas its nonfunction-blocking counterpart, J1B5, had only minimal effect (Figure 5D).

Collectively, our studies of mouse sperm binding to cultured mouse cells indicate that cells that express $\alpha 6$ and $\beta 1$ at their surface bind significantly more sperm than their $\alpha 6$ - or $\beta 1$ -deficient counterparts. Moreover, simultaneous surface expression of both $\alpha 6$ and $\beta 1$ is required for the sperm-binding phenotype. These cultured cell studies

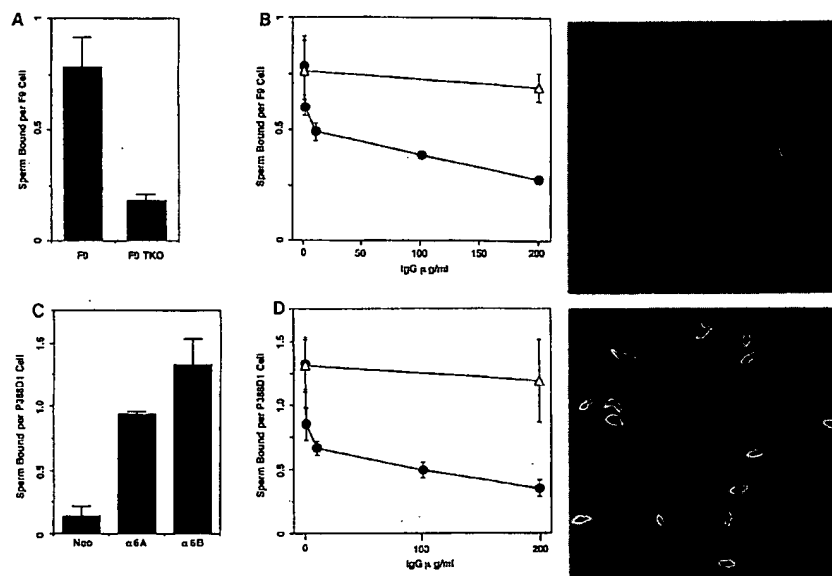


Figure 5. Sperm Binding to Cultured Cells

(A-D) The indicated cells were incubated with sperm, washed, and scored for sperm binding. In (B) and (D), F9 and $\alpha 6B$ -transfected P388D1 cells, respectively, were treated with the indicated amount of GoH3 (closed circles) or J1B5 (open triangles). 500 cells were counted for each experimental condition. Each experiment was repeated at least three times with similar results. Representative experiments are shown. (E and F) Sperm binding to P388D1 cells transfected with neo (E) or $\alpha 6B$ (F) was visualized with the aid of fluorescent probes as described in Experimental Procedures. Examples of sperm heads are indicated with arrows.

therefore corroborate our conclusion that the mouse egg integrin $\alpha 6\beta 1$ is critically involved in sperm binding.

Fertilin Is Involved in $\alpha 6\beta 1$ -Mediated Sperm Binding

We next investigated the role of mouse fertilin β in $\alpha 6\beta 1$ -mediated sperm binding. A synthetic peptide corresponding to the 14-residue predicted binding loop of the mouse fertilin β disintegrin domain (Wolfsberg et al., 1995) inhibited mouse sperm binding to eggs (Figure 6A) and to $\alpha 6B$ -transfected P388D1 macrophages (Figure 6B). Cyclized (M14-C) and linear (M14-L) versions of the peptide appeared equally effective. A corresponding 13-residue peptide analog of the PII snake disintegrin, atrolysin E (HTE-RGD), a peptide that prevents platelet aggregation (Fox et al., 1995), had no effect.

Since sperm only bind avidly to cells that express $\alpha 6\beta 1$ at their surface, and since a peptide analog of mouse fertilin β inhibits this binding, it appears highly likely that fertilin mediates sperm binding to $\alpha 6\beta 1$. To explore this possibility, we tested the effects of the mouse fertilin β peptide on the ability of anti- $\alpha 6$ MABs to bind to the egg surface. If the mouse fertilin β peptide and GoH3 both inhibit sperm binding through the same or neighboring sites on $\alpha 6\beta 1$, then the fertilin peptide might compete with GoH3 for binding to eggs. As shown in Figure 7B, the mouse fertilin β peptide significantly reduced binding of GoH3 to eggs. Staining with J1B5, the nonfunction-blocking anti- $\alpha 6$ MAB, was not affected (Figure 7E), and the snake disintegrin

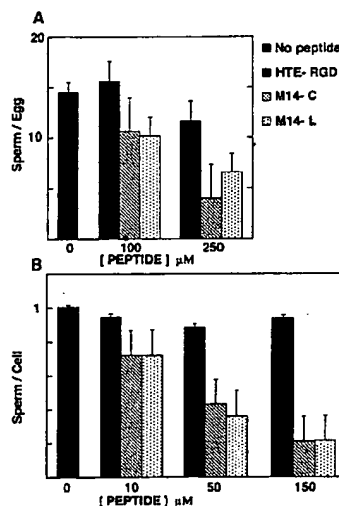


Figure 6. Effect of Disintegrin Peptide Analogs on Sperm Binding

Peptide analogs of the mouse fertilin β (M14-C and M14-L) and atrolysin E (HTE-RGD) disintegrin loops were freshly dissolved in DMSO to a concentration of 25 mM and then diluted in embryo culture media to the indicated concentrations. All samples were normalized to contain 2.5% DMSO. Peptide solutions were added to eggs (A) or $\alpha 6B$ -transfected P388D1 cells (B) for 30 min at 37°C. Sperm were then added and incubated for 1 hr at 37°C, at which time samples were processed for counting as described in Figures 3 and 5, respectively. Each experiment was repeated three times. A representative experiment is shown.

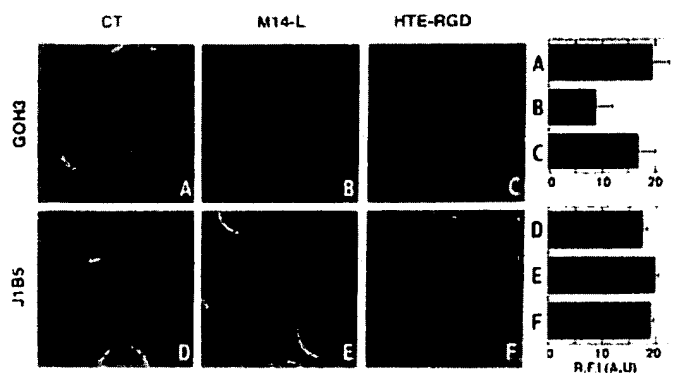


Figure 7. Effect of Disintegrin Peptide Analogs on the Reactivity of Anti- $\alpha 6$ MABs with Mouse Eggs

The indicated peptides (nomenclature as in Figure 6) were dissolved in DMSO to a concentration of 50 mM and then diluted in embryo culture media to 500 μ M. CT, no peptide control. Peptides were added to eggs for 30 min at 4°C. Next, ascites fluid (1:1000 dilution) containing the GoH3 (top panels) or J1B5 (bottom panels) MABs was added, and the incubations continued for a further hour on ice. Samples were washed three times with cold medium and then processed for immunofluorescence. Egg fluorescence intensities (far right) were quantitated as described in Experimental Procedures. 20 eggs were used for each measurement, and data are presented \pm SEM. R.F.I., relative fluorescence intensity. The experiment was repeated twice with similar results.

peptide, HTE-RGD, had no effect (Figures 7C and 7F). Collectively, these results indicate that the fertilin disintegrin domain is involved in $\alpha 6\beta 1$ -mediated sperm binding.

Discussion

Fertilin is a sperm surface protein involved in binding and fusion with the egg plasma membrane. As the sequence of fertilin contains a disintegrin domain (Blöbel et al., 1992), we hypothesized that an integrin on the egg plasma membrane serves as a sperm receptor. In the present study, we tested this hypothesis. Based largely on antibody inhibition and somatic cell transfection experiments, we conclude that the integrin $\alpha 6\beta 1$ serves as a murine sperm receptor and that both $\alpha 6A$ and $\alpha 6B$, isoforms that vary only in their cytoplasmic tail sequences, can support this function. Our finding that a specific egg integrin, $\alpha 6\beta 1$, serves as a murine sperm receptor has three major implications. First, it demonstrates a novel role for the integrin $\alpha 6\beta 1$. Second, it offers a plausible general mechanism for adhesion among mammalian gametes. Third, it suggests a novel, and perhaps widely used, mechanism of cell-cell recognition and signaling.

Novel Role for the Integrin $\alpha 6\beta 1$

The mouse egg integrin $\alpha 6\beta 1$ plays a key role in binding mouse sperm. Prior to this study, the only known ligands for $\alpha 6\beta 1$ had been members of the laminin family of extracellular matrix proteins (Albelda and Buck, 1990; Hynes, 1992; Mercurio and Shaw, 1991; Rousselle and Aumailley, 1994). Neither mouse eggs (Hierck et al., 1993) nor mouse sperm (data not shown) express laminin, and mouse sperm do not express $\alpha 6\beta 1$ (data not shown). Our results thus suggest that binding between mouse sperm and $\alpha 6\beta 1$ on the egg plasma membrane is mediated by a direct interaction with a coreceptor on sperm, as opposed to via an indirect interaction through laminin. Mounting evidence suggests that the sperm coreceptor for $\alpha 6\beta 1$ is the disintegrin domain of fertilin (see next section). Hence, our results point to a novel role for $\alpha 6\beta 1$ as a mediator of an important cell-cell interaction, as opposed to its established roles

in cell-matrix interactions. Moreover, since integrins are well-known to transduce signals (Hynes, 1992; O'Toole et al., 1994; Sastry and Horwitz, 1993), binding of a sperm ligand to $\alpha 6\beta 1$ on the egg could be a key event that initiates development of the mouse embryo.

Mouse sperm bind to mouse somatic cells that express $\alpha 6\beta 1$ (Figure 5). Since the $\alpha 6$ transfectants used in this study express human $\alpha 6$ in complex with mouse $\beta 1$, it is clear that the $\alpha 6$ subunit does not need to be of murine origin to fulfill this role. Two lines of evidence suggest, however, that the ability of $\alpha 6\beta 1$ to serve as a murine sperm receptor may have additional requirements and be regulatable. The first is that human cells that express human $\alpha 6$ /human $\beta 1$ (Delwel et al., 1993) do not bind mouse sperm. The second is that the requirements for sperm and laminin binding to $\alpha 6$ -transfected P388D1 cells and the relative abilities of $\alpha 6A$ and $\alpha 6B$ to fulfill these roles appear to be different (Figure 5; Shaw and Mercurio, 1994).

Sperm-Egg Binding Based on Fertilins and Integrins

Several lines of evidence suggest that the coreceptor for mouse egg $\alpha 6\beta 1$ is a sperm fertilin. First, all fertilins analyzed, from spermatogenic cells from a variety of species, possess a disintegrin domain (Heinlein et al., 1994; Ramarao et al., 1995; Wolfsberg et al., 1993, 1995). Second, a peptide analog of the guinea pig fertilin β disintegrin domain inhibits guinea pig fertilization *in vitro* (Myles et al., 1994). Third, a peptide analog of the mouse fertilin β disintegrin domain inhibits sperm binding to both mouse eggs and to $\alpha 6\beta 1^+$ somatic cells (Figure 6). Fourth, the same peptide significantly diminishes the reactivity of GoH3, a function-blocking anti- $\alpha 6$ MAB, with the egg surface (Figure 7). Fifth, a complex has been observed containing mouse fertilin β and $\alpha 6$ (E. A. C. A. and A.-P. J. H., unpublished data). Hence, it appears highly likely that binding between mouse sperm and eggs is mediated, at least in part, by a sperm fertilin engaging the integrin $\alpha 6\beta 1$ on the egg. In this regard, it is interesting that Sertoli cells express $\alpha 6\beta 1$ (but not laminin) at sites of interaction with developing spermatids (Salanova et al., 1995) and that

developing spermatids express fertilins (Blobel et al., 1990; Wolfsberg et al., 1993, 1995).

All mammalian eggs analyzed (mouse, hamster, human) express integrins at their surface (Fusi et al., 1993; Hlerck, et al., 1993; Sutherland et al., 1993; Tarone et al., 1993). Although differences have been reported as to the specific integrin subunits present on eggs from a particular species, there is complete agreement that the integrin $\alpha\beta 1$ is present on the surface of eggs from all mammalian species analyzed. Prior studies that have addressed the role of particular integrins in sperm-egg binding have concluded that RGD-containing peptides inhibit binding of human and hamster sperm to golden hamster eggs. Whether different species of mammalian eggs use RGD-dependent (e.g., hamster) and RGD-independent (e.g., mouse) integrins as sperm receptors, or whether the results of Bronson and Fusi (1990) reflect the noted promiscuous nature of golden hamster eggs (Yanagimachi, 1994) remains to be determined. Nevertheless, since sperm and eggs from all mammalian species analyzed express, respectively, fertilins and integrins on their surfaces, it seems probable that binding between sperm fertilins and egg plasma membrane integrins is a general mechanism for interaction among mammalian gametes. We have recently discovered that there are several other fertilin homologs, in addition to fertilin α and β , expressed in spermatogenic cells of guinea pigs and mice (Wolfsberg et al., 1995). If, as suggested, fertilins bind integrins, then it remains to be determined for each mammalian species, which sperm fertilin(s) binds to which egg integrin(s).

We propose that binding between fertilins and integrins may provide a general mechanism for adhesion among mammalian gametes. Whether the presence of fertilin homologs (B. Podbilewicz, personal communication) and integrins (Hynes and Marcantonio, 1989; Lallier et al., 1994) in lower eukaryotes implies a role for these molecules in other mating reactions (Folz et al., 1993) remains to be determined.

Conclusions and Perspectives

In conclusion, we have shown that $\alpha\beta 1$ on the egg plasma membrane serves as a mammalian sperm receptor and that it can fulfill this function in somatic cells as well. We have also provided evidence that the sperm ligand that interacts with $\alpha\beta 1$ is fertilin. Fertilin is the prototype of a growing and widely distributed family (ADAMs) of membrane proteins that possess an integrin ligand domain with the disintegrin motif (Wolfsberg et al., 1993, 1995). Hence, a direct interaction between ADAMs and integrins might represent a novel type of cell-cell interaction used not only for sperm-egg binding, but also for other important cell-cell recognition (Ruiz et al., 1993) and signaling events.

Experimental Procedures

Mice

Eggs were obtained from 8- to 10-week-old female ICR mice. Sperm were obtained from male ICR retired breeders. Mice were acclimated for at least 5 days prior to use.

Egg Isolation

Germinal vesicle (GV) stage oocytes were isolated from the ovaries of superovulated mated female mice. Females were superovulated by injection of 5 IU of pregnant mare serum (PMS) followed 48 hr later by 5 IU of human chorionic gonadotropin (HCG). Approximately 40 hr after HCG injection, mice were sacrificed and ovarian tissue was removed and dissociated with a 150 mesh tissue sieve (Sigma, St. Louis, MO) in embryo culture medium (Spindle, 1980) containing 1 μ g/ml isobutyl methyl xanthine (IBMX) to prevent premature germinal vesicle breakdown. GV stage oocytes were then collected and washed in embryo culture medium. After "softening" for 3 min at 37°C in embryo culture medium containing 10 μ g/ml chymotrypsin, zona pellucidae were removed by passage through a narrow bore pipette. GV stage oocytes were returned to normal embryo culture medium and matured overnight in vitro (Calarco, 1991). Alternatively, ovulated egg masses containing mature eggs were used. Ovulated egg masses were removed from the oviducts of unmated superovulated females 12 hr after HCG injection and placed in embryo culture medium containing hyaluronidase (250 μ g/ml) for 5 min at 37°C to detach cumulus cells. After washing and removing cumulus cells, zona pellucidae were softened and mechanically removed as described above.

Sperm Isolation

Sperm were isolated from the cauda epididymis of male retired breeders. Dissected caudae were sliced open with a scalpel, and the sperm were released with gentle agitation into embryo culture medium (Spindle, 1980) supplemented with 2.5 mM pyruvate and 3% bovine serum albumin. Sperm were allowed to capacitate for 90 min in this medium. Prior to capacitation, sperm were 5%-10% acrosome reacted. Following capacitation, sperm were 50%-75% acrosome reacted (see next section).

Assessment of Acrosomal Status

The acrosomal status of free sperm (pre- and postcapacitation) or sperm bound to tissue culture cells was determined using Coomassie brilliant blue as described in Aarons et al. (1991). The acrosomal status of sperm bound to eggs was determined using FITC-conjugated peanut agglutinin essentially as described in Tao et al. (1993). Sperm-egg samples were counterstained with propidium iodide (Molecular Probes, Eugene, OR) and observed by confocal microscopy.

Antibodies

Antibodies were obtained from the following sources: polyclonal rabbit pan-integrin antiserum (Lenny III), Dr. C. Buck; polyclonal rabbit $\alpha\beta 3$ antiserum, Drs. J. Gailit and E. Ruoslahti; polyclonal rabbit antiserum to a cytoplasmic domain peptide of $\alpha 3$ integrin subunit, Dr. R. Hynes; rat MAb J1B5 (prepared versus human $\alpha 6$), Dr. C. Damsky; rat MAb 5H10 (versus mouse $\alpha 5$), Pharmingen Incorporated (San Diego, CA). Like J1B5, rat MAb GoH3 was prepared versus human $\alpha 6$. MAbs J1B5 and GoH3 were purified from hybridoma culture supernatants by binding to protein G columns. The columns were washed with PBS, eluted with 20 mM glycine (pH 2.7) directly into 1 M Tris (pH 9), and then concentrated and washed three times with PBS using Centricon 30 concentrators (Amicon, Beverly, MA). We used preparations of GoH3 shown to block adhesion of murine EHS tumor cells to laminin.

Peptides

The peptides used in this study correspond to the predicted binding loops of the disintegrin domains of mouse fertilin β (Wolfsberg et al., 1995) and the PII snake disintegrin atrolysin E (Hite et al., 1994). They are referred to as M14 and HTE-RGD, respectively. Their sequences are as follows: M14, CRLAQDEADVTEYC; HTE-RGD, CRVSRGDRNDDTC. HTE-RGD was the gift of Dr. J. Fox. Peptides were synthesized by the University of Virginia Biomedical Research Facility on a Bioscience 9600 using FMOC amino acid protection. The M14 peptide was used in both linear (M14-L) and cyclized (M14-C) forms. The HTE-RGD peptide used was cyclized. Cyclization was conducted using standard procedures. All peptide sequences were confirmed by mass spectroscopy. For the M14 peptide, the cysteine residue in the middle of the fertilin β disintegrin loop (Wolfsberg et al., 1995) was replaced with an alanine to prevent disulfide bond formation at this position during cyclization. For the HTE-RGD peptide, the central residues, MVD, were changed to RGD. Whereas the authentic (MVD) peptide

does not inhibit platelet aggregation, the RGD-substituted peptide does (Fox et al., 1995).

Immunofluorescence Detection of Integrins

Indirect immunofluorescence labeling of live and fixed/permeabilized eggs was performed as described (Sutherland et al., 1991). Eggs were obtained as described above. Primary polyclonal rabbit antibodies were visualized with rhodamine-conjugated goat anti-rabbit IgG (Calbiochem, San Diego, CA); primary rat monoclonal antibodies were visualized with rhodamine-conjugated goat anti-rat IgG (Calbiochem, San Diego, CA). Fluorescence images were acquired with a scanning confocal microscope (Bio-Rad MRC600, Bio-Rad, Cambridge, England). Paired phase-contrast images were acquired simultaneously with a transmitted light detector. For quantitation of fluorescence intensity (Figure 7), images were acquired in the same focal plane and with the same microscope settings. Background fluorescence was subtracted, and the average pixel intensity per egg determined.

PCR

mRNA was isolated from 1000 mature mouse eggs from ovulated egg masses using the Micro Fasttrack kit (Invitrogen, San Diego, CA). Single-stranded cDNA was synthesized using Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) and random hexamer primers. The cDNA was ethanol precipitated and resuspended in 60 μ l of sterile water; 3 μ l was used per 50 μ l of PCR reaction. Reaction conditions and the primers used for α 1, α 3, α 5, α 6, α 7, and β 1 were exactly as described previously (Sutherland et al., 1993). Other primers were as follows: α 2, 5'-TCTGCGTGTGGACATCAGTTTGA-3' and 5'-GATAACCCCTGTCGGTACTTCTGCG-3' (1140 bp); α v, 5'-GAAGG-ACAATGTCTG-3' and 5'-CCCGCTTGGTGATGAG-3' (790 bp); β 3, 5'-CTGGTGTGTACCAGTATGCCAAG-3' and 5'-TGTTGAGGCAGG-TGGCATTGAAGG-3' (393 bp); β 4, 5'-GGACACACCTGTGAGATCA-CTA-3' and 5'-ACAGTATTTCAGCAGAGCAGCAG-3' (374 bp); β 5, 5'-ACTTGGAGAACATCCGGAGC-3' and 5'-TTGAAGCTGTGCGACT-CTGTC-3' (235 bp); and β 6, 5'-GCTTGGCTCCCGGCTGGC-3' and 5'-AGTTAATGGCAAAATGTGCT-3' (190 bp). Amplified products were separated on a 2% agarose gel using a 1 kb DNA ladder (GIBCO-BRL, Gaithersburg, MD) for size comparison. Primers for α v were from Drs. J. Adelman and C. Buck; primers for β 5 and β 6 were from Dr. D. Sheppard.

Cell Cultures

F9 and F9 TKO cell monolayers were cultured in DMEM (Mediatech, Washington, D. C.) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine. For binding experiments, the cells were detached and dissociated with trypsin-EDTA for 5 min at 37°C, plated at approximately 10^4 cells/ml/cm², and used for binding assays 12 hr later. P388D1 cells and transfectants thereof were grown as monolayers in DMEM supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine, and 1 mg/ml G418 (Geneticin, GIBCO-BRL). For binding experiments, the cells were detached by scraping, pelleted, and washed. The cells were then plated at approximately 10^4 cells/ml/cm² and used for binding assays after 3 hr. All cells were grown in a 37°C incubator.

Flow Cytometry

F9 cells were detached from culture dishes with Ca²⁺/Mg²⁺-free PBS containing 2 mM EDTA and washed and resuspended in PBS. Cells were labeled on ice with 200 μ g/ml GoH3 for 45 min, washed three times with ice-cold PBS, labeled on ice for 45 min with secondary antibody, washed three times with cold PBS, and analyzed on a Becton-Dickinson fluorescence-activated cell sorter using Lysis II software. GoH3 was visualized using fluorescein-conjugated goat anti-rat Fab2 IgG fragments (Cappel, West Chester, PA). The fluorescence value reported is the median relative fluorescence intensity for 10,000 events. All cell lines were analyzed using the same sensitivity settings on the instrument.

Sperm-Egg Binding and Fusion Assays

Eggs were isolated as described above and preloaded with 1 μ M Hoechst 33342 for 30 min to allow detection of fusing sperm (Conover and Gwatkin, 1988). Antisera were heat inactivated by incubation at

56°C for 45 min. Eggs were placed in antisera or purified IgG 30 min prior to in vitro insemination. Antibodies were present throughout sperm-egg incubations. Sperm were added to 100 μ l incubation droplets containing eggs under mineral oil to produce a final concentration of approximately 0.5×10^6 – 1.0×10^6 sperm/ml. After 60 min, eggs were washed three times with embryo culture medium. This yielded approximately 15–25 sperm bound per untreated egg. Phase-contrast images were recorded to score sperm binding. Fluorescence images were recorded to score fusion, evidenced by fluorescent labeling of sperm DNA from Hoechst 33342 present in preloaded eggs (Conover and Gwatkin, 1988). Only mature eggs that were arrested in meiotic metaphase II prior to fertilization and that were completely free of zona fragments were analyzed. Unless stated, data are presented \pm SEM.

Sperm-Cultured Cell Binding Assays

F9 and P388D1 cells were plated in 6-well culture plates and incubated with antibodies 30 min prior to sperm addition. Sperm were then added to a final concentration of approximately 0.5×10^6 – 1.0×10^6 /ml, 2 ml per well, and allowed to bind for 45 min at 37°C in a CO₂ incubator. Cells were washed three times with embryo culture medium and three times with normal PBS. Sperm binding was visualized with an inverted phase-contrast microscope. The number of sperm bound per cell was scored within 30 min of washing. Data are presented \pm SEM. For fluorescence images (Figures 5E and 5F), cells were prelabeled with Syto 11 (Molecular Probes, Eugene, OR). Following sperm binding, samples were washed, fixed with 4% paraformaldehyde, stained with propidium iodide, and viewed by confocal microscopy.

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Prindle, Kathleen - LAW

From: Mulhollan, Michael [michael.mulhollan@unisys.com]

Sent: Thursday, September 07, 2006 1:09 PM

To: prindlek@amgen.com

Subject: Ticket #5443607 - Discovery Team Mtg room change "Occurs the third Wednesday of every month"

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Please do not reply to this email, as this account is not constantly monitored and I will be out of office for several days.

Thank you,

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